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Impact of DNA mismatch repair proteins deficiency on number and ratio of lymph nodal metastases in colorectal adenocarcinoma

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ABSTRACT

Background: Approximately 15 % of colorectal adenocarcinomas (CRCs) are characterized by an altered expression of DNA mismatch repair (MMR) proteins (i.e. MMR deficiency [MMRd]). Lymph node ratio (LNR) represents one of the most important prognostic markers in non-advanced CRCs. No significant data are available regarding LNR distribution depending on MMR status.

Purpose of the study: The aim of the present work was to compare pathological and clinical characteristics of MMRd tumors versus MMR proficient (MMRp) cases. Particular attention was paid to how these molecular subgroups relate to the LNR.

Materials and methods: A mono-Institutional series of 1037 consecutive surgically treated stage I-IV CRCs were retrospectively selected and data were obtained from pathological reports. Cases were characterized for MMR/MSI status by means of immunohistochemistry or for microsatellite instability (MSI) analysis.

Results: MMRd/MSI tumors (n = 194; 18.7 %) showed significant differences in comparison to MMRp lesions for sex (female prevalence 50.5 % vs 40.7 %; $p = 0.013$), age (74.2 vs 69.2; $p < 0.001$), location (right side; $p < 0.001$), diameter (larger than MMRp; $p < 0.001$), growth pattern (expansive pattern of growth; $p < 0.001$), peri- ($p = 0.0002$) and intra-neoplastic ($p = 0.0018$) inflammatory infiltrate, presence of perineural invasion ($p < 0.001$), stage (lower stage at presentation; $p < 0.001$), grade (higher prevalence of high-grade tumors; $p < 0.001$), and LNR (lower; $p < 0.001$).

Conclusions: MMRd/MSI tumors are a distinct molecular CRC subtype characterized by a significantly lower LNR in comparison to MMRp lesions. These data further support the prognostic impact of MMRd/MSI status in early-stage CRCs.

1. Introduction

Colorectal adenocarcinoma (CRC) is nowadays the third cancer for incidence in men and the second in women and is the second cause of neoplastic death worldwide [1,2]. The incidence is higher in Western countries and is increasing in middle-low income countries [1].

DNA mismatch repair (MMR) system is involved in the repair of base pairing errors, insertions, or deletions during DNA replication and

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primarily includes four essential proteins: MLH1, PMS2, MSH2 and MSH6 [3]. Loss of function of this protein complex leads to an increased mutational burden. Microsatellite instability (MSI) is the *epiphenomenon* of MMR deficiency (MMRd). Microsatellites are repetitive sequences distributed throughout the human genome, which are subject to the accumulation of mutations. MMRd/MSI status can be evaluated by immunohistochemistry (IHC) for MLH1, PMS2, MSH6 and MSH2 and/or by PCR-based molecular assays for MSI [3]. IHC analysis and molecular tests are highly concordant, both with high sensitivity and specificity, in rare cases discordances are observed due to preanalytical factors and within the frame of mucinous histotype [4,5].

Deficit in the MMR system is present in 15 % of CRCs when considering Lynch syndrome-associated CRCs and sporadic tumors [3]. The genetic hallmark of Lynch syndrome is the germline mutation in one of the MMR genes, while in sporadic CRCs the deficit in MMR system is frequently caused by methylation of *MLH1* gene promoter with its subsequent transcriptional silencing or it can be due to a somatic mutation in one of the genes. Furthermore, approximately 70 % of sporadic MMRd/MSI CRCs are associated with a ^{V600E}*BRAF* mutation, which is absent in Lynch syndrome [3].

Regardless of their sporadic or inherited nature, MMRd/MSI CRCs are associated with peculiar clinco-pathological features. They are generally right-sided, and they frequently have a poorly differentiated/mucinous histology with a heavy intra-tumoral (especially intra-epithelial) lymphocytic infiltration and a Crohn-like reaction at the invasion front. MMRd/MSI is also a well-established prognostic and predictive factor. MMRd/MSI CRCs are associated with a better stage-adjusted prognosis in comparison with MMR proficient (MMRp), with a lower risk of recurrence and a longer overall survival [6]. MMRd/MSI is believed to be predictor of resistance to 5-fluoruracile based chemotherapy. In the metastatic setting, MMRd/MSI identifies a subset of CRCs which are responsive to immune check-point inhibitors therapy. Of note, in 2020 the anti-PD1 antibody pembrolizumab was approved as first-line therapy in MMRd/MSI advanced unresectable or metastatic CRCs [7].

^{V600E}*BRAF* gene mutation is present in a significant portion of sporadic MMRd/MSI CRCs. This molecular feature generally confers a poor prognosis to CRCs. Several studies investigated the relationship between *BRAF* mutations and MMR status with contrasting results. Most studies concluded that MMRd *BRAF* mutated (*BRAFmut*) CRCs have poorer prognosis than MMRd/MSI *BRAF* wild type (*BRAFwt*) CRCs and similar or better prognosis than MMRp *BRAFwt* CRCs [8–12,22]. However, limited data are available on the histopathological landscape of ^{V600E}*BRAF*-mutated CRCs.

CRCs has several prognostic factors, including stage, histological subtype, grade, lymph node ratio (LNR), lymph-vascular invasion and perineural infiltration [13]. LNR can be defined as the ratio between positive nodes and total nodes examined. Many studies demonstrated that a lower LNR is associated with better prognosis, and it has a higher prognostic value than other known prognostic factors lymph nodes-correlated, like lymph node yield (LNY) and total number of positive lymph nodes [14–20].

The aim of this work was to compare pathological and clinical characteristics of MMRd/MSI CRCs with MMRp neoplasms, with particular attention to LNR.

2. Materials and methods

2.1. Case selection and clinic-pathological characterization

All CRCs undergoing surgical excision between January 2016 and June 2019 at Padua University Hospital were included, regardless of the execution of neoadjuvant therapy. Surgical resection specimens were processed according to a local standardized protocol following WHO and TNM guidelines. For colon adenocarcinomas, only cases with 12 or more lymph nodes isolated from the perivisceral fat were included. In rectal adenocarcinomas, in which patients usually undergo neoadjuvant therapies, only cases with a minimum number of 6 lymph nodes isolated from the perivisceral fat were included. All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Declaration of Helsinki (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/").

The information recorded included age and sex of patient, maximum diameter (cm) and site of neoplasm. Site of tumor was recorded as right colon, transverse colon, left colon or sigma-rectum.

Information regarding age and sex of patient, maximum diameter (cm), neoadjuvant therapy, tumor regression grade (TRG) and site of neoplasm were collected from pathologic and clinical records. Data on neoadjuvant therapy and TRG according to Mandard [21] were collected only for rectal adenocarcinomas.

Two experienced pathologists (MF and FZ) jointly evaluated the original reports according to the morphologic World Health Organization 4th edition criteria. Cases were jointly re-evaluated in case of histological discordances/data missing. The cases were subclassified in 3 groups according to their histotype: not otherwise specified (NOS; also including micropapillary and serrated histotypes), mucinous and rare variants (i.e., medullary, signet ring, adenosquamous histotypes).

Other histological features were evaluated, including pattern of growth, inflammatory infiltrate intra- and peritumoral, perineural infiltration, lymph-vascular invasion and grade. Grade was attributed according to the WHO 4th edition four-tiered grading system, based on the percentage of gland formation.

Pathological stage (pN and pT) was evaluated according to TNM classification of Malignant Tumors UICC 8th edition for those diagnosed after 2017.

The LNR was calculated as the ratio of metastatic nodes and total lymph nodes isolated from the perivisceral fat. The LNR was then subclassified according to the system proposed by Rosenberg et al. [16] as LNR 0 (no lymph node metastasis), LNR 1 ($0.1 \leq LNR \leq 0.17$), LNR 2 ($0.18 \leq LNR \leq 0.41$), LNR 3 ($0.42 \leq LNR \leq 0.69$) and LNR 4 ($LNR \geq 0.7$).

2.2. Immunohistochemistry

IHC was performed using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle upon Tyne, UK) in the BOND-MAX system (Leica Biosystems). Four-μm-thick FFPE sections were incubated with the following primary antibodies: MLH1 (clone ES05; Dako), PMS2 (clone EP51; Dako), MSH2 (clone FE11; Dako), MSH6 (clone EP49; Dako). Samples were defined as MMRd when at least one of the four proteins resulted negative according to the GIPAD-SIAPeC criteria [3].

2.3. Microsatellite instability

Samples in which IHC analysis was inadequate or resulted undetermined were further analyzed by adopting Titano MSI test (Diatech Pharmacogenetics). Briefly, the extracted DNA derived from tumor and corresponding normal mucosa were analyzed with the MSI Titano kit following the manufacturer's instructions. The Titano MSI kit evaluates MSI status by multiplex amplification with fluorescent primers and subsequent DNA length fragment analysis on an automated sequencer. Starting from 20 ng of extracted DNA, this assay can detect variation in the number of repetitive sequences for $n = 10$ different microsatellite loci (*BAT25*, *BAT26*, *D2S123*, *D17S250*, *D5S346*, *BAT40*, *D18S58*, *NR21*, *NR24* and *TGFβRII*) by comparing tumor and corresponding normal tissue profiles generated from the capillary electrophoresis.

2.4. BRAF mutational analysis

All cases with combined loss of MLH1 and PMS2 or MSI were profiled for ^{V600E}*BRAF* mutation. *BRAF* mutational profiling was carried out on formalin-fixed paraffin-embedded (FFPE) samples from primary tumors

and/or paired metastases by means of Sanger Sequencing, Sequenom MassArray technology (Myriapod Colon status; Diatech Pharmacogenetics), or by the Easy Pgx Real-Time *BRAF* kit (Diatech Pharmacogenetics).

2.5. Statistical analysis

In all patients, data were compared using Kruskal-Wallis test (continuous variables) or Fisher's test (categorical data). All tests were 2-sided and a p-value less than 0.05 was considered statistically significant. Statistical analysis was performed using R 3.3 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

All the main clinical and histopathological features of the considered series are summarized in Table 1. A total of 1037 cases were included in the present work, subdivided in 843 (81.3 %) MMRp and 194 (18.7 %) MMRd/MSI neoplasms. Among MMRd/MSI tumors, 74 (38.1 %) cases were *BRAF*wt, 107 (55.1 %) were *BRAF*mut and in 13 (6.8 %) cases *BRAF* status was not assessed. MMRp cancers received neoadjuvant therapy in 132 cases (15.7 %; most rectal adenocarcinomas), while none was treated before surgery in the MMRd/MSI group.

3.1. Clinical and macroscopic data

Patients of the MMRp group were 500 (59.3 %) males and 343 (40.7 %) females, while in the group of MMRd/MSI neoplasms there were 96 (49.5 %) males and 98 (50.5 %) females. Mean age was 69.2 ± 12.9 years old in MMRp patients and 74.2 ± 13.0 years old in MMRd/MSI patients.

There was a positive association between female sex and MMRd/MSI ($p = 0.013$) and a more advanced age observed among MMRd/MSI patients ($p < 0.001$).

MMRp cancers were located more frequently in the rectum-sigmoid colon (n = 423; 50.2 %), followed by right colon (n = 304; 36 %), while MMRd/MSI cancers were predominantly in the right colon (n = 150; 77.3 %), and in the transverse colon (n = 21; 11.4 %). MMRd/MSI cancers were more often right-sided ($p < 0.001$). The mean maximum diameter was respectively 4.1 ± 2.0 cm for MMRp group and 5.3 ± 2.4 for MMRd/MSI group, and MMRd/MSI tumors were associated with a greater diameter ($p < 0.001$).

Fig. 1 represents the distribution of clinical and macroscopic parameters across the molecular subtypes.

3.2. Histologic features

As regards histological features (Fig. 2), MMRp were predominantly of NOS histotype (n = 786; 93.2 %), followed by mucinous histotype (n = 54; 6.4 %) and rare variants were identified (0.4 %). MMRd/MSI CRCs were more frequently of NOS histotype (n = 154; 79.4 %), followed by mucinous (n = 38; 19.6 %) and other rare histotypes (1.0 %). Mucinous histology was more frequent among MMRd/MSI CRCs than MMRp neoplasms (p = 0.011).

Most of the MMRp tumors had an infiltrative pattern of growth (n = 494; 58.6 %) while MMRd/MSI tumors had a predominant expansive growth pattern (n = 104; 53.6 %). This difference was statistically significant, with the expansive growth pattern being more frequent in the MMRd/MSI group than in MMRp group ($p < 0.001$).

Lymphocytic infiltration was present in most cases of MMRp CRCs and it was more frequently of low grade, either peri-tumoral (n = 478; 56.7 %) or intra-tumoral (n = 584; 69.3 %). Like in the MMRp group, most MMRd/MSI cases had low-grade lymphocytic infiltration, either peri-tumoral (n = 102; 52.6 %) or intra-tumoral (n = 132; 68 %). Regardless of the grade of the inflammatory infiltrate, MMRp CRCs had peritumoral inflammatory infiltrate in 73.9 % (n = 623) and intra-

Table 1
Clinical, macroscopic, and histologic features collected for MMRp and MMRd/MSI CRCs. Data about MMRd/MSI are further divided in *BRAF*wt and *BRAF*mut cases.

	MMRp	MMRd/MSI	MMRd/MSI *BRAFwt*	MMRd/MSI *BRAFmut*
Sex				
M	500 (59.3 %)	96 (49.5 %)	41 (55.4 %)	47 (43.9 %)
F	343 (40.7 %)	98 (50.5 %)	33 (44.6 %)	60 (56.1 %)
Age (years)	69.2 ± 12.9	74.2 ± 13.0	68.8 ± 15.0	78.3 ± 8.5
Neoadjvant Therapy				
Yes	132 (15.7 %)	0 (0 %)	0 (0 %)	0 (0 %)
No	711 (84.3 %)	194 (100 %)	74 (100 %)	107 (100 %)
Site of cancer				
Right colon	304 (36.1 %)	150 (81.5 %)	54 (73 %)	88 (82.2 %)
Transverse colon	24 (2.8 %)	21 (11.4 %)	6 (8.1 %)	11 (10.3 %)
Left colon	92 (10.9 %)	8 (4.3 %)	4 (5.4 %)	4 (3.7 %)
Sigma-rectum	423 (50.2 %)	15 (8.2 %)	10 (13.5 %)	4 (3.7 %)
Mean maximum diameter (cm)	4.1 ± 2.0	5.3 ± 2.4	5.8 ± 2.5	4.9 ± 2.3
Histotype				
NAS	786 (93.2 %)	154 (79.4 %)	64 (86.5 %)	81 (75.7 %)
Mucinous	54 (6.4 %)	38 (19.6 %)	10 (13.5 %)	25 (23.3 %)
Other	3 (0.4 %)	2 (1 %)	0 (0 %)	1 (1 %)
Pattern of growth				
Expansive	340 (40.3 %)	104 (53.6 %)	40 (54.1 %)	57 (53.3 %)
Mixed	9 (1.1 %)	4 (2.1 %)	1 (1.4 %)	3 (2.8 %)
Infiltrative	494 (58.6 %)	86 (44.3 %)	33 (44.6 %)	47 (43.9 %)
Peritumoral lymphocyte infiltrate				
Absent	220 (26.1 %)	35 (18 %)	15 (20.3 %)	20 (18.7 %)
Low grade	478 (56.7 %)	102 (52.6 %)	37 (50 %)	59 (55.1 %)
High grade	145 (17.2 %)	56 (28.9 %)	21 (28.4 %)	28 (26.2 %)
Not available	0 (0 %)	1 (0.5 %)	1 (1.4 %)	0 (0 %)
Intratumoral lymphocyte infiltrate				
Absent	182 (21.6 %)	29 (14.9 %)	13 (17.6 %)	14 (13.1 %)
Low grade	584 (69.3 %)	132 (68 %)	49 (66.2 %)	75 (70.1 %)
High grade	77 (9.1 %)	32 (16.5 %)	11 (14.9 %)	18 (16.8 %)
Not available	0 (0 %)	1 (0.5 %)	1 (1.4 %)	0 (0 %)
Vascular invasion				
Absent	256 (30.4 %)	60 (30.9 %)	30 (40.5 %)	26 (24.3 %)
Present	587 (69.6 %)	133 (68.6 %)	43 (58.1 %)	81 (75.7 %)
Not available	0 (0 %)	1 (0.5 %)	1 (1.4 %)	0 (0 %)
Perineural invasion				
Absent	416 (49.3 %)	130 (67 %)	51 (68.9 %)	69 (64.5 %)
Present	427 (50.7 %)	63 (32.5 %)	22 (29.7 %)	38 (35.5 %)
Not available	0 (0 %)	1 (0.5 %)	1 (1.4 %)	0 (0 %)
Grade				
G1	101 (12 %)	18 (9.3 %)	7 (9.5 %)	8 (7.5 %)
G2			29 (39.2 %)	33 (30.8 %)

(*continued on next page*)

Table 1 (*continued*)

	MMRp	MMRd/MSI	MMRd/MSI *BRAFwt*	MMRd/MSI *BRAFmut*
	589 (69.9 %)	67 (34.5 %)		
G3	110 (13 %)	107 (55.2 %)	37 (50 %)	66 (61.7 %)
G4	1 (0.1 %)	2 (1 %)	1 (1.4 %)	0 (0 %)
Not available	42 (5 %)	0 (0 %)	0 (0 %)	0 (0 %)
Stage				
I	149 (17.7 %)	37 (19.1 %)	14 (18.9 %)	22 (20.6 %)
II	267 (31.7 %)	92 (47.4 %)	37 (50 %)	46 (43 %)
III	326 (38.7 %)	58 (29.9 %)	20 (27 %)	35 (32.7 %)
IV	101 (12 %)	7 (3.6 %)	3 (4.1 %)	4 (3.7 %)
pT				
1	46 (5.5 %)	6 (3.1 %)	2 (2.7 %)	3 (2.8 %)
2	136 (16.1 %)	35 (18 %)	14 (18.9 %)	21 (19.6 %)
3	503 (59.7 %)	106 (54.6 %)	39 (52.7 %)	59 (55.1 %)
4	158 (18.7 %)	47 (24.2 %)	19 (25.7 %)	24 (22.4 %)
pN				
0	425 (50.4 %)	132 (68 %)	53 (71.6 %)	69 (64.5 %)
1	254 (30.1 %)	43 (22.2 %)	15 (20.3 %)	26 (24.3 %)
2	164 (19.5 %)	19 (9.8 %)	6 (8.1 %)	12 (11.2 %)
Lymph node examined	24.1 ± 11.8	29.4 ± 21.4	33.8 ± 24.5	25.5 ± 10.7
LNR				
0	460 (54.6 %)	135 (69.6 %)	55 (74.3 %)	70 (65.4 %)
1	222 (26.3 %)	43 (22.2 %)	15 (20.3 %)	26 (24.3 %)
2	116 (13.8 %)	10 (5.2 %)	2 (2.7 %)	7 (6.5 %)
3	38 (4.5 %)	6 (3.1 %)	2 (2.7 %)	4 (3.7 %)
4	7 (0.8 %)	0 (0 %)	0 (0 %)	0 (0 %)

tumoral infiltrate in 78.4 % (n = 661) of cases, while MMRd/MSI CRCs had peritumoral inflammatory infiltrate in 81.5 % (n = 158) and intratumoral infiltrate in 84.5 % (n = 164) of cases. As expected, inflammatory infiltrate was associated with MMRd/MSI, both peri-tumoral ($p = 0.0002$) and intra-tumoral ($p = 0.0018$).

Vascular invasion was present in 587 cases (69.6 %) of MMRp CRCs and in 133 MMRd/MSI neoplasms (68.6 %), with no statistically significant difference.

Perineural infiltration was less frequent in MMRd/MSI CRCs than in MMRp cancers ($p < 0.001$). In fact, perineural infiltration was present in 50.7 % (n = 427) and absent in 49.3 % (n = 416) of MMRp CRCs, while it was present in 32.5 % (n = 63) and absent in 67 % (n = 130) of MMRd/MSI CRCs.

With regard to grading (according to WHO 2010 criteria), in the MMRp group 589 cases (69.9 %) were G2, followed by G3 (n = 110; 13 %) and G1 (n = 101; 12 %), with only one G4 cancer (0.1 %). On the other hand, in MMRd/MSI group grade was G3 in 107 cases (55.2 %), G2 in 67 cases (34.5 %), G1 in 18 (9.3 %) and only 2 cancers were G4 (1 %).

MMRd/MSI neoplasms were more frequently of high-grade (G3-G4) ($p < 0.001$). In fact, high-grade neoplasms were 56.2 % (n = 109) in MMRd/MSI group and 13.1 % (n = 111) in MMRp group.

3.3. Staging data and LNR

MMRp neoplasms were predominantly stage III (n = 326; 38.7 %), followed by stage II in 267 cases (31.7 %), stage I in 149 (17.7 %) and stage IV in 101 (12 %). MMRd/MSI were instead more frequently stage II (92 cases; 47.4 %), followed by stage III (n = 58; 29.9 %), stage I (n = 37; 19.1 %) and only rare stage IV cancers were found (n = 7; 3.6 %). The statistical analyses demonstrated that stage at presentation was statistically lower in MMRd/MSI group ($p < 0.001$).

MMRp CRCs were pT1 in 5.5 % (n = 46), pT2 in 16.1 % (n = 136), pT3 in 59.7 % (n = 503) and pT4 in 18.7 % (n = 158). MMRd/MSI CRCs were pT1 in 3.1 % (n = 6), pT2 in 18 % (n = 35), pT3 in 54.6 % (n = 106) and pT4 in 24.2 % (n = 47). No statistical differences were found between MMRd/MSI neoplasms and MMRp cancers regarding pT.

As regards lymph nodes involvement (Fig. 3), pN was 0 in 50.4 % (n = 425), 1 in 30.1 % (n = 254) and 2 in 19.5 % (n = 164) in the group of MMRp, with a mean lymph node yield (LNY) of 24.1 ± 11.8. Instead, pN was 0 in 68 % (n = 132), 1 in 22.2 % (n = 43) and 2 in 9.8 % (n = 19) in the group of MMRd, with a mean LNY of 29.4 ± 21.4. LNY was statistically higher in MMRd/MSI group ($p < 0.001$).

In the MMRp group, LNR was 0 in 54.6 % (n = 460), 1 in 26.3 % (n = 222), 2 in 13.8 % (n = 116), 3 in 4.5 % (n = 38) and 4 in 0.8 % (n = 7). In the MMRd/MSI group, LNR was 0 in 69.6 % (n = 135), 1 in 22.2 % (n = 43), 2 in 5.2 % (n = 10), 3 in 3.1 % (n = 6), with no case being LNR4 in the MMRd/MSI group. The LNR was statistically lower in MMRd/MSI ($p < 0.001$), with a not statistically higher prevalence of LNR2/3 cases among *BRAF*mut cases.

When considering only right-sided CRCs, in the MMRd/MSI group, LNR was 0 in 67.3 % (n = 101), 1 in 24 % (n = 36), 2 in 4.7 % (n = 7), 3 in 4.0 % (n = 6), while in the MMRp group, LNR was 0 in 51.6 % (n = 157), 1 in 28.9 % (n = 88), 2 in 15.1 % (n = 46), 3 in 3.7 % (n = 11), 4 in 0.7 % (n = 2). Thus, the LNR was statistically lower in MMRd/MSI ($p = 0.002$). In right-sided MMRp CRCs, the mean LNY was 26.5 ± 15.6, while in right-sided MMRd CRCs the mean LNY was 30.1 ± 23.3; no statistical differences were found between MMRd/MSI neoplasms and MMRp cancers regarding LNY.

4. Discussion

The present study examined a mono-Institutional case series of CRCs, investigating the clinico-pathologic features associated with MMRd/MSI tumors to explore the relationship between MMRd/MSI status and LNR in CRCs. For this purpose, we excluded from the analysis cases with an insufficient number of lymph nodes retrieved, aware that most rectal cancers underwent resection after neoadjuvant radiochemotherapy and that the number of lymph nodes retrieved is low after preoperative treatment [23,24]. Of note, there was no MMRd/MSI CRC located in the sigmoid colon and rectum [26], and none of the patients with an MMRd/MSI tumor received neo-adjuvant therapy.

Two subgroups of MMRd/MSI CRC were also considered: the *BRAF*wt and the *BRAF*mut tumors, which are expression of two different mechanisms leading to MSI, the first one having the same profile of Lynch syndrome (LS)-related CRCs. Recognition of LS was not the goal of this paper, however the rate of MMRd/MSI *BRAF*wt tumors in our study is 7 %. Being the incidence of Lynch syndrome 2–4 % in unselected series of CRCs [25], half of the MMRd/MSI *BRAF*wt in our series would be related to germline MMR deficiency.

In line with previous works, MMRd/MSI CRCs were more frequent in women than men ($p = 0.013$) and in older patients ($p < 0.001$), were more frequently right sided ($p < 0.001$) and had a higher mean tumor diameter ($p < 0.001$).

Unsurprisingly, MMRd/MSI CRCs were also more frequently of mucinous histotype ($p = 0.011$), were characterized by a heavier peri- ($p = 0.0002$) and intra-tumoral inflammatory infiltrate ($p = 0.0018$) and by a higher grade (G3-G4) at presentation ($p < 0.001$). Vascular invasion rate was not statistically different between MMRp and MMRd/MSI tumors. Moreover, this study further demonstrated that MMRd/MSI CRCs were associated with favourable prognostic factors. In fact, MMRd/MSI CRCs showed statistically more frequent expansive growth

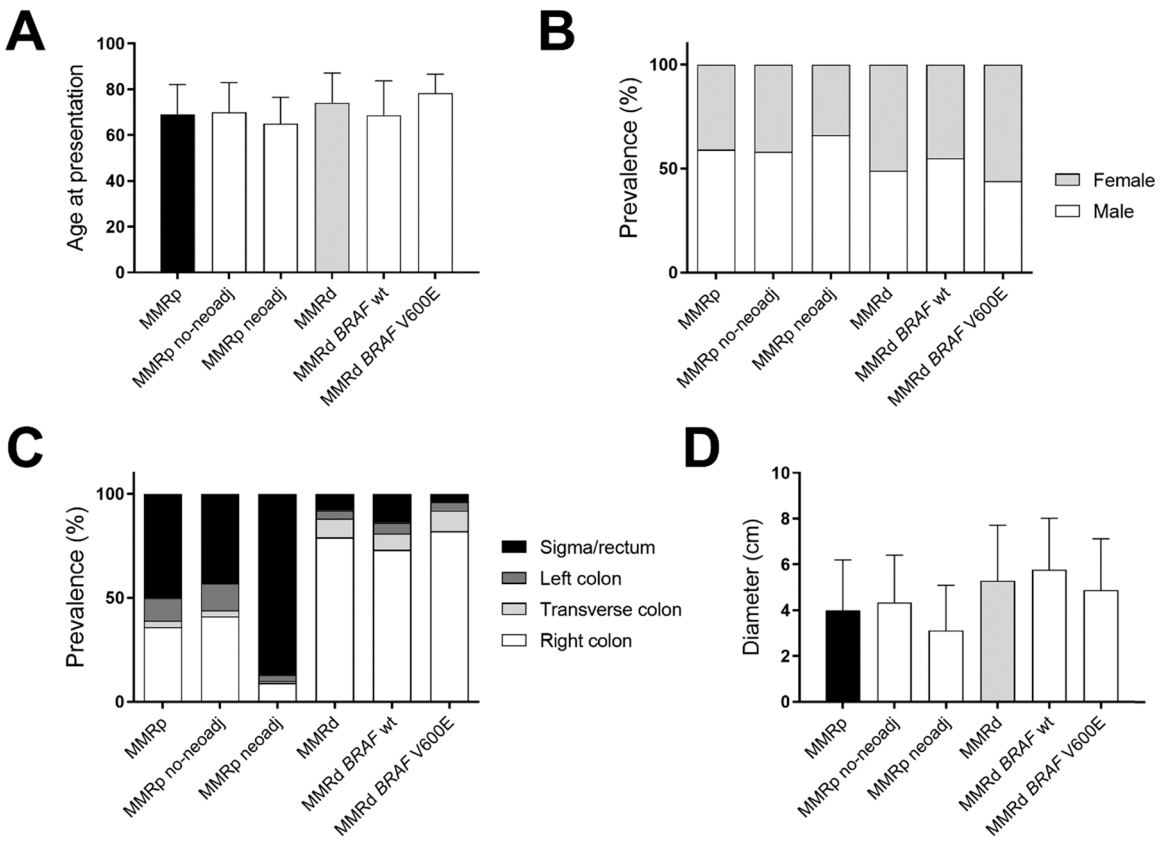

Fig. 1. Summary and comparison of clinical and macroscopic data for MMRp cancers (subdivided according to execution or not of neoadjuvant therapy) and MMRd/MSI neoplasms (subdivided according to *BRAF* status). (**A**) Age at presentation, showing a statistical higher age for MMRd/MSI (74.2 years) than for MMRp (69.2 years). (**B**) Sex: female sex was statistically higher for MMRd/MSI group (n = 98; 50.5 %) than for MMRp group (n = 343; 40.7 %). (**C**) Site of neoplasms: MMRd/MSI cancers were localized more frequently than MMRp cancers in the right colon, with a statistically significant difference ($p < 0.001$). MMRp neoplasm were more frequently in sigma-rectum. (**D**) Mean diameter of cancer: MMRd/MSI neoplasms had statistically higher mean tumor diameter (5.3 cm) than MMRp cancers (4.1 cm).

pattern ($p < 0.001$), lower stage at presentation ($p < 0.001$) and less frequent peri-neural infiltration ($p < 0.001$).

Several studies have demonstrated the prognostic role of LNR (i.e. the ratio between positive lymph nodes and total nodes examined) and its superior prognostic value in comparison with LNY and total number of positive nodes [14–20].

Higher LNY is associated with a better prognosis. Different factors influence the number of lymph nodes harvested: LNY is higher in right colon neoplasms, poorly differentiated, bigger cancers, tumors with marked inflammatory infiltrate and MMRd/MSI neoplasm [19,20, 27–33]. Accordingly, in this study more lymph nodes were harvested in MMRd/MSI neoplasms than in MMRp neoplasms ($p < 0.001$). Colorectal tumors exhibiting prominent antitumor immune reactions, like MMRd/MSI tumors, have been associated with hyperplastic changes in regional lymph nodes. Lymph nodes draining these tumors are consequently larger and more detectable, explaining the relation between MMRd/MSI status and lymph node yield in the resection specimen [34].

Lee et al. [19] conducted an observational study of 1585 patients with CRCs with a median follow-up of 27.1 months and demonstrated that higher LNR was associated with poorer disease-free survival (DSF) and overall survival (OS). Ceelen et al. [14] reviewed 16 studies, which overall included 33,984 stage III CRCs and found that LNR is a more efficient prognosticator than the total number of positive nodes. Rosenberg et al. [16] analysed 3026 CRCs and demonstrated through a multivariate analysis that LNR and N stage are both independent prognostic factors, but LNR emerged as the most reliable prognostic factor. In our study, LNR was lower in MMRd/MSI than MMRp neoplasms ($p < 0.001$). This phenomenon has to be attributed to the lower lymph node involvement rate and to the higher lymph node harvest in this group (24.1 ± 11.8 versus 29.4 ± 21.4). It is known that the number of harvested lymph nodes in right colon is greater than in the left side and most MMRd CRCs are located in the right colon. The comparative analysis between the right-sided MMRd/MSI and MMRp revealed a statistically significant higher LNR in MMRd/MSI CRCs and a higher LNY, without reaching statistical significancy.

Furthermore, because no statistically significant difference in LNR was observed between *BRAF*wt and *BRAF*mut MMRd/MSI tumors, it appears that MSI itself is associated with lower lymph nodes metastatic rate in CRC, regardless of the *BRAF* mutational status.

In conclusion, MMRd/MSI identifies a subgroup of CRCs with distinct clinico-pathologic features, reflecting a distinctive molecular background and tumor biology. Microscopic features and staging data that emerged from our results overall point out to a favorable prognostic value of MMRd/MSI in CRCs. From this point of view, the balance between adverse (greater tumor diameter, mucinous histotype and higher grade) and favorable (heavier tumoral inflammatory infiltrate and expansive growth pattern) prognostic factors in MMRd/MSI tumors, tends overall toward a low chance of lymph node metastasis.

Recently, the American Society of Clinical Oncology (ASCO) updated guidelines for adjuvant treatment of stage II colon cancer [35]. Adjuvant Chemotherapy may be offered to patients with stage IIA (i.e., T3) colon cancer with clinical and histological high-risk features, considering pros and cons of the adjuvant treatment within a multidisciplinary approach.

In this context, the results of our study can be helpful in this decision-making process, because indicate that MMRd/MSI is not only a favorable prognostic biomarker but is also associated with lower risk of

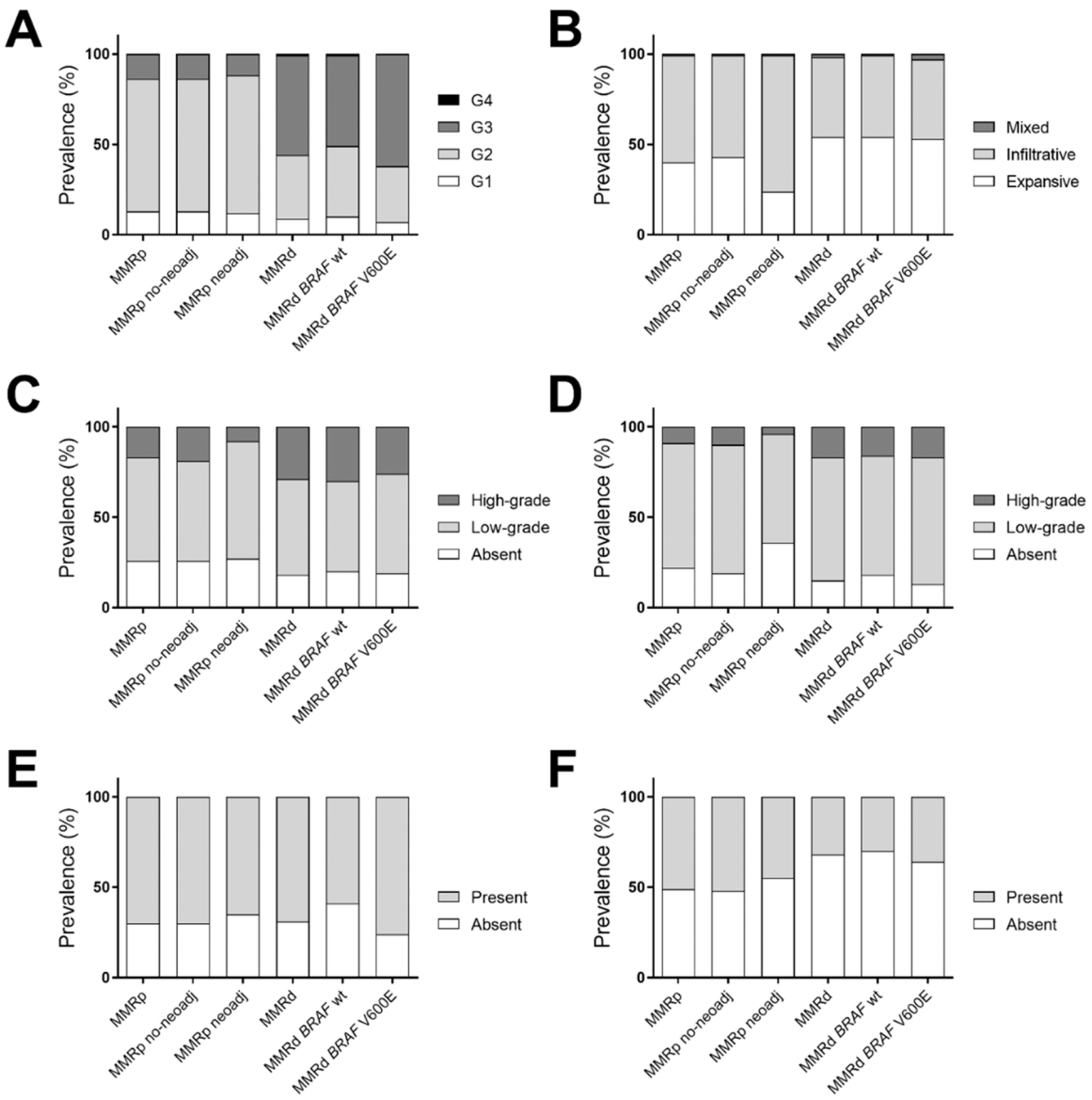

Fig. 2. Summary and comparison of histological features for MMRp cancers (subdivided according to execution or not of neoadjuvant therapy) and MMRd/MSI neoplasms (subdivided according to *BRAF* status). (**A**) Grade: grade was reported according to the 4th edition of WHO classification of gastrointestinal neoplasms as G1 to G4 according to the percentage of gland formation. MMRd/MSI group had a higher prevalence of high-grade neoplasms than MMRp group. (**B**) Pattern of growth: it was reported as expansive, infiltrating or mixed, with a statistically higher expansive growth pattern for MMRd/MSI group. (**C**) Peri-tumoral inflammatory infiltrate: it was reported as absent or present, and if present it was divided in low or high grade. MMRd/MSI group had more frequently peri-tumoral inflammatory infiltrate than MMRp ($p = 0.0002$). (**D**) Intra-tumoral inflammatory infiltrate: it was reported as absent or present, and if present it was divided in low or high grade. MMRd/MSI group had more frequently intra-tumoral inflammatory infiltrate than MMRp ($p < 0.001$). (**E**) Vascular invasion: no statistically significant difference was found between MMRp and MMRd/MSI neoplasms. (**F**) Perineural invasion: it was present more frequently in MMRd/MSI group than MMRp group ($p < 0.001$).

lymph nodes dissemination. However, larger multi-Institutional efforts should further dissect the heterogeneous CRC landscape in order to perform a better patient stratification to optimize the therapeutic management of the disease.

Ethics approval statement

All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Declaration of Helsinki (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/).

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Authors' statement

MF, GS, ELDU, SP, and APDT conceptualized and designed the study. FZ, VA, SB, DS, MB, MS, SL, FB, CM, MSc, QRB collected samples and provided clinical data. FZ, VA, SB, CM, APDT provided histopathology expertise. FZ, VA, GS, and MF performed the data analysis. MF, GS, ELDU, SP, and APDT contributed to interpretation of the results. FZ, VA, and GS wrote the first draft of the manuscript, and all authors critically revised the manuscript. All authors approved the final version of the manuscript.

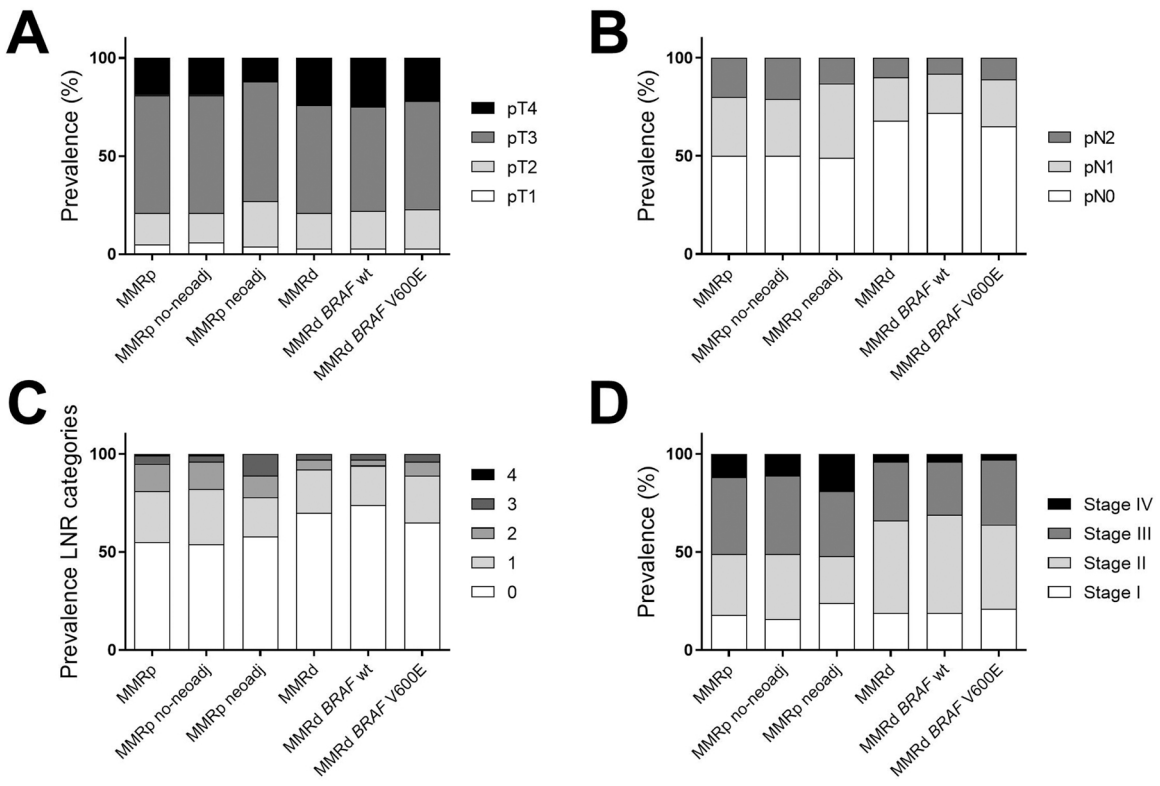

Fig. 3. Summary and comparison of staging data and LNR for MMRp cancers (subdivided according to execution or not of neoadjuvant therapy) and MMRd/MSI neoplasms (subdivided according to BRAF status). (**A**) pT: no statistical differences were found in pT between MMRd/MSI neoplasms and MMRp cancers. (**B**) pN: it was lower in MMRd/MSI group. (**C**) LNR: LNR, i.e. the ratio between positive lymph nodes and total nodes harvested, was statistically lower in MMRd/MSI group than MMRp group. (**D**) Pathological stage: MMRd/MSI neoplasms were at lower stage at diagnosis than MMRp group.

Declaration of Competing Interest

None related to the current work.

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